Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 7, No. 8, pp. 1021-1026, 1997

0960-894X/97 $17.00 + 0.00

PII: S0960-894X(97)00161-3

SYNTHESIS AND CREATINE KINASE INHIBITORY ACTIVITY OF NON-HYDROLYZABLE ANALOGS OF PHOSPHOCREATINE

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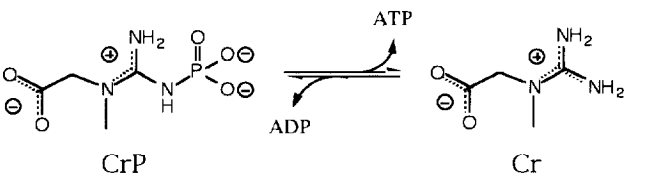
Abstract: The first hydrolytically stable phosphocreatine (CrP) analogs with a CH_2-P isostere in place of the NH-P linkage have been synthesized and found to inhibit creatine kinase (CK) with potencies in the low mM range. The closest structural analog of CrP, **2**, was found to have a K_i (1.4 mM) near the K_m of CrP (2.5 mM), making it the most potent known reversible inhibitor of CK. © 1997 Elsevier Science Ltd.

CK catalyzes the reversible transfer of a phosphoryl group between CrP and ADP (Scheme 1).[1] The creatine kinase shuttle system connects sites of ATP production with sites of ATP utilization. This is possible through the localization of CK isoforms to different cellular compartments. Indeed, the role of the CK/CrP system in cellular energy regulation appears to be as a mechanism for storage, transport and rapid generation of ATP in cells with fluctuating and sudden energy demands, the conversion of CrP to ATP being driven by local ATP/ADP ratios and pH.[2]



Scheme 1

Recently, the CK system has been implicated in cellular transformation processes[3] as well as the replication of some viruses.[4] Creatine (Cr) analogs were shown to be effective antitumor and antiviral agents in a variety of in vitro and in vivo model systems.[5] The compounds that exhibited antitumor activity[6] were those which could build up to high intracellular levels in their phosphorylated forms and ultimately replace Cr/CrP in the cells with a less efficient energy surplus.[7] This same study reported that administering some Cr analogs in

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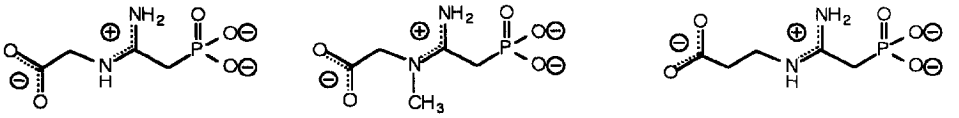
their phosphorylated forms also imparted antitumor activity, though through a mechanism which is not fully understood. The compounds that most closely resembled the structure of phosphocreatine were found to be the most potent. In addition, it has also been shown that PEP and CrP in red blood cells augments FceRI-mediated activation of phospholipase C (PLC) by a mechanism that, at least in part, is independent of their ability to generate ATP.[8] As yet, however, there have been no reports in the literature of hydrolytically stable CrP analogs. Here we detail the synthesis and enzyme inhibitory results of a series of amidinomethylphosphonate compounds in which the C-NH-P unit of known phosphocreatine analogs has been replaced by C-CH_2-P (**1–3**) (Figure 1).

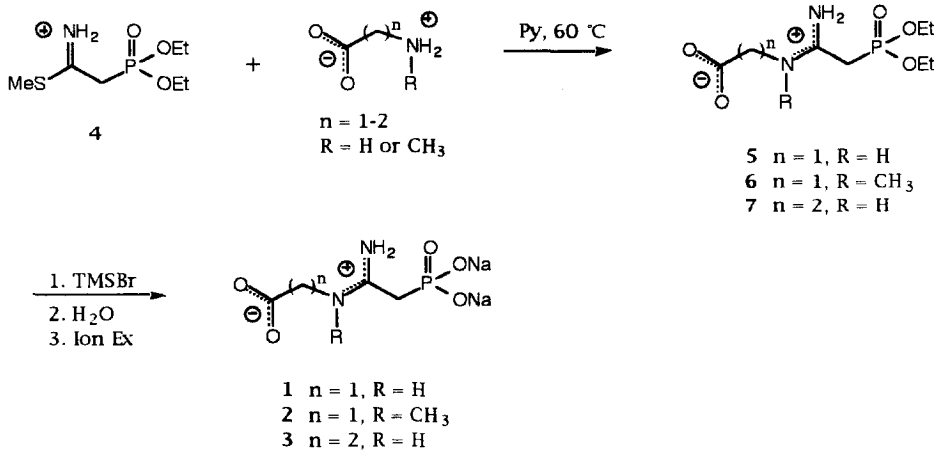


1 **2** **3**

Figure 1

Chemistry

A very concise synthesis was developed for the preparation of this class of compounds starting with 2-(diethylphosphono)-S-methylthioacetamidinium iodide (**4**), an intermediate used previously for the preparation of carbamoyl phosphate synthetase II inhibitors.[9] This compound, known to readily react with alkylamines to produce amidinomethylphosphonates, was treated with amino acids in pyridine to obtain the protected analogs



4

5 n = 1, R = H
6 n = 1, R = CH_3
7 n = 2, R = H

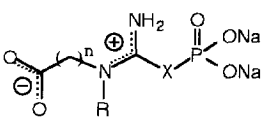
1 n = 1, R = H
2 n = 1, R = CH_3
3 n = 2, R = H

Scheme 2

in good yield (Scheme 2).[10] Subsequent conversion of the diethyl phosphonates to the phosphonic diacids was accomplished with bromotrimethylsilane followed by aqueous hydrolysis.[11] Purification of the final compounds with ion exchange chromatography resulted in isolation of the pure compounds as their disodium salts. It is significant to note that these compounds display no propensity toward the lactamization to creatinine-like structures that often hampers preparations of Cr analogs.[12]

Results and Discussion

Reported herein is the first synthesis and enzymatic analysis of a short series of hydrolytically stable phosphocreatine analogs. The inhibition assay results reflect the success of the amidinomethylphosphonate group as a replacement of the phosphoguanidine subunit of CrP and its analogs (Table 1). Though the K_m

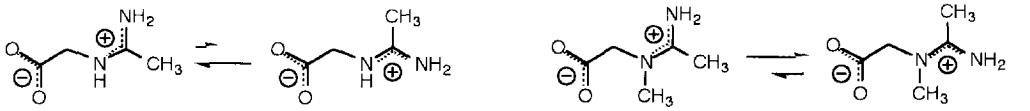


#	n	R	K_i (mM) X = CH_2	K_m (mM) X = NH
1	1	H	5.7 ± 0.1	n.a.
2	1	CH_3	1.4 ± 0.1	2.5
3	2	H	37 ± 20	n.a.

Table 1

values for two of the corresponding substrates were not obtainable due to exceedingly slow conversion rate in the presence of CK, the comparison of **2** with its substrate counterpart, CrP, is very favorable. In addition, Lineweaver-Burke analysis of **1** revealed that it displays purely competitive behavior versus CrP.

As in the case of the substrates, a net improvement in binding is clearly observed with the introduction of a phophoryl group (K_m Cr = 30 mM, K_m CrP = 2.5 mM), the importance of which is most evident when **2** is compared with completely inactive **9**.[13] Not only does this represent a case where a phosphonate isostere is a functional replacement for a phosphoramide, but **2** is the most potent reversible inhibitor of CK reported to date. The similarity of the K_i of **2** to the K_m of CrP is interesting to note since the expected geometric rigidity of the phosphonoguanidine unit is presumably lost in the amidinomethylphosphonate. This result also suggests that the solvation parameters inside and outside the active site for NH and CH_2 are balanced. One may envision that the entropy loss incurred by the more flexible phosphonate is made up for by the improved desolvation capacity of the more lipophilic CH_2. Similar effects have been reported for transition state analog inhibitors of thermolysin[14] and matrix metalloproteinases[15] where a CH_2-P subunit confers similar or even greater potency



8 **9**

Figure 2

than NH-P. The thermolysin result was even anticipated through the use of free energy perturbation calculations.[16]

^{1}H NMR spectra taken in D_2O for these compounds show roughly the same s-*cis*/s-*trans* amidine ratios described earlier by Wang[17] for amidines **8** and **9** (Figure 2). Integrations of the alpha-phosphonomethylene proton signals of **1** and **3** confirm that these compounds exist almost entirely in the s-*trans* conformation. Inhibitor **2**, however, slightly favors the s-*cis* amidine conformation by a 1.2/1 ratio, in reasonable agreement with Wang's observation of 2.3/1 for **9**. This result suggests that the enzyme is presented with a mixture in which only about half of the inhibitor molecules are in the presumed s-*trans* active conformation at any given time.[18] Further study is needed to determine if this is relevant to the potency of **2**.

In conclusion, a series of creatine kinase inhibitors were prepared with a CH_2 unit in place of the NH of the natural substrate, phosphocreatine. Inhibition studies revealed that in at least one case these compounds are competitive with respect to phosphocreatine, and that the inhibitor which most closely resembles CrP, **2**, binds with a K_i value (1.4 mM) below the respective substrate's K_m (2.5 mM).

Determination of K_is

Enzyme assays were conducted in a manner similar to that described by Lamprecht, et al.,[19] in which a coupled system consisting of hexokinase and G-6-P dehydrogenase uses the ATP generated in the CK reaction to convert $NADP^+$ to NADPH. The assays were run at 37 °C and the appearance of NADPH was monitored on a UV/visible spectrophotometer at a wavelength of 340 nm. Rabbit muscle creatine kinase (Sigma, Type I, C-3755) was used and concentration determined spectrophotometrically at 280 nm using the extinction coefficient 7.1×10^4 as reported. The K_i values were determined from V_0/V_i vs [I] plots ($V_0/V_i = [I]/K_i(1 + [S]/K_m) + 1$) with five or more different concentrations of I over a range of at least $1/2(K_i)$ - $5(K_i)$. Inhibition was determined for the direction of ATP formation only. Lineweaver–Burk analysis was used to determine the mode of observed inhibition versus CrP.

Acknowledgement

This work was funded by the Repligen Corporation.

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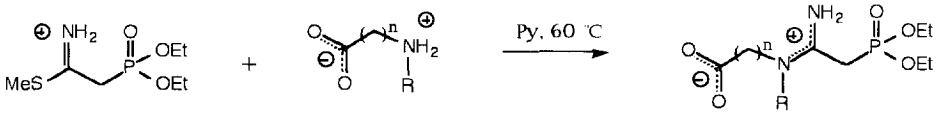
10. In a typical experimental procedure:
Diethyl *N*-(carboxymethyl)amidinomethylphosphonate (**5**): 1.0165 g (2.90 mmol) of **4** was added to a stirred mixture of 0.2213 g (2.95 mmol) of glycine, 1.0 mL of dichloromethane and 2.0 mL of pyridine. The mixture was stirred overnight and then filtered to remove solids. After rinsing with dichloromethane, hexanes was added to the filtrate until it was persistently cloudy, and the mixture was placed in a 5 °C refrigerator overnight. The solid was removed by filtration and air dried on the filter after thorough rinsing with cold dichloromethane. The white solid weighs 0.5897 g (44.3%).



4

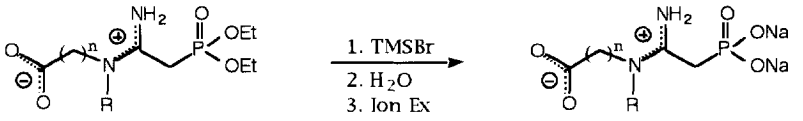
n = 1-2
R = H or CH_3

5 n = 1, R = H
6 n = 1, R = CH_3
7 n = 2, R = H

	Yield	1H NMR	31P NMR
5	44%	1.35 (t, 6H, J = 7.0 Hz), 3.38 (d, 2H, J = 22.2 Hz), 3.94 (s, 2H), 4.25 (dq, 4H, $J_1 = J_2$ = 7.1 Hz)	21.2 (anti) 20.5(syn)
6	99%	1.35 (t, 6H, J = 7.1 Hz), 3.22 (s, 2H, CH_3 syn), 3.36 (s, 1H, CH_3 anti), 3.50 (d, 1.3H, J = 22.5 Hz, CH_2P syn), 3.56 (d, 0.7H, J = 22.5 Hz, CH_2P anti), 4.18-4.33 (m, 4.7H), 4.43 (s, 1.3H, CH_2N syn)	20.3 (anti) 20.1 (syn)
7	76%	1.33 (t, 6H, J = 7.1 Hz), 2.74 (t, 2H, J = 6.1 Hz), 3.14 (d, 2H, J = 22.1 Hz), 3.59 (t, 2H, J = 6.0 Hz), 4.21 (dq, 4H, $J_1 = J_2$ = 7.2 Hz)	20.9

11. In a typical experimental procedure:
N-(Carboxymethyl)amidinomethylphosphonic acid, disodium salt (**1**): Bromotrimethylsilane (1.5 equiv.) was added to diethyl *N*-(carboxymethyl)amidinomethyl-phosphonate (1.0 equiv.) and the mixture was stirred at room temperature overnight. The excess TMSBr was evaporated under vacuum. The residue was redissolved in of water and stirred at room temperature for 4 h. The mixture was then lyophilized to dryness. The solid was redissolved in water, loaded onto an anion exchange column (Dowex 1X8-200, HCO3- form) (18 mm x 9.4 cm) and eluted with a linear gradient from 100% water to 1 M TBK (TBK = triethylammonium bicarbonate, prepared by mixing 139 mL of triethylamine in 1 L of water and

bubbling CO2 into the mixture until the pH reaches 8.6). The fractions with product were identified by spotting them on a silica plate and staining with ninhydrin. The ones which had material that stained more darkly than triethylamine and ran with TBK were combined and lyophilized to dryness. The solid was lyophilized twice more from 2 mL of water each time to ensure that no TBK remained. The solid was dissolved in water, loaded onto a cation exchange column (Dowex 50X8-100, Na+ form) (10 mm x 9 cm) and eluted with water. Fractions containing product were identified as before, combined and lyophilized to dryness.



5 n = 1, R = H
6 n = 1, R = CH_3
7 n = 2, R = H

1 n = 1, R = H
2 n = 1, R = CH_3
3 n = 2, R = H

	Yield	1H NMR	31P NMR	Formula	Anal. Calcd	Anal. Found
1	51%	2.99 (d, 2H, J = 19.4 Hz), 4.01 (s, 2H, CH_2N of anti), 4.16 (s, v. minor, CH_2N of syn)	10.1 (syn) 11.1 (anti)	$C_4H_7N_2O_5PNa_2$ •$3H_2O$	C, 16.33; H, 4.45; N, 9.52	C, 16.65; H, 4.52; N, 9.37
2	5%	2.79 (d, 1.1H, J = 18.7 Hz, CH_2P syn), 2.94 (d, 0.9H, J = 19.0 Hz, CH_2P anti), 3.15 (s, 1.7H, CH_3 syn), 3.32 (s, 1.3H, CH_3 anti), 4.09 (s, 0.9H, CH_2N anti), 4.30 (s, 1.1H, CH_2N syn)	9.6 (syn) 10.0 (anti)	$C_5H_9N_2O_5PNa_2$ •H_2O	C, 22.07; H, 4.08; N, 10.29	C, 22.42; H, 4.45; N, 9.64
3	38%	2.71 (t, 2H, J = 6.7 Hz), 2.95 (d, 2H, J = 19.2 Hz), 3.64 (t, 2H, J = 6.6 Hz)	10.4 (syn) 11.1 (anti)	$C_5H_9N_2O_5PNa_2$ •$2H_2O$	C, 20.70; H, 4.52; N, 9.65	C, 21.10; H, 4.86; N, 9.44

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(*Received in USA* 14 *February* 1997; *accepted* 13 *March* 1997)